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Characterization of Dual and Homeostatic Chemokine Expression and Response to Bacterial Infection in Large Yellow Croaker, *Larimichthys crocea*

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Authors' Contribution
JL and CW conceived and designed the experiments. TZ and X.D performed the experiments. JL and WL analyzed the data. T.Z and XY contributed reagents/materials/ analysis tools. JL and TZ wrote the paper. TZ and XY collected field material and processed the samples.

Key words
Anti-infection, *Larimichthys crocea*, CC chemokines, Immune mechanism, Bacterial infection.

ABSTRACT

Chemokines are cytokines which can induce leukocyte activation and migration that are secreted as a part of an immune response. However, while the role of chemokines has been described extensively in mammals, little is known regarding such immune responses in fish species. The current study reports on the gene expression and molecular characteristics of three members of the CC family of chemokines: 1) dual chemokine ligand CCL17; 2) homeostatic chemokines ligand CCL21; and 3) CCL24 in response to bacterial challenge in large yellow croaker (*Larimichthys crocea*). The results revealed that the immunological effect of CCLs in the croaker is weaker than that of other fish, and revealed the infectivity of the large yellow croaker. Thus, these findings could help to develop methods of disease resistance in the aquaculture industry.

INTRODUCTION

Large yellow croaker (*Larimichthys crocea*) is a unique local fish species in China and is widely distributed in the north from the Yellow Sea south, by the East China Sea, the Taiwan Strait, south to Leizhou Peninsula, and east in the South China Sea. Due to its high yield, large yellow croaker has become an important marine aquaculture fish species in China. Over the past decade, through continuous effort and exploration, scientists have successfully achieved the ability to artificially breed large yellow croaker in cages. Due to the rapid development of artificial cultivation, the scale of aquaculture production has continued to expand, becoming the largest marine aquaculture cage species in China. However, with the growth of the culture scale, improvement of the breeding density, pollution of the aquaculture environment, and lack of farming management, problems associated with disease have become the main factors restricting the sustainable and stable development of the large yellow croaker. Therefore, by elucidating the molecular basis of large yellow croaker immunization and revealing its molecular mechanism of immunity or disease resistance, we can 1) deepen the knowledge of the lower vertebrate immune system using fish as a representative model; and 2) provide a theoretical basis for disease control in large yellow croaker to facilitate the cultivation of disease-resistant varieties of this species.

Chemokines are cytokines which can induce leukocyte activation and migration in response to immune activation. Chemokine superfamily members have been identified and well-studied regarding their function in both humans and mouse models (Rollins, 1997; Yoshie *et al*., 2001; Zlotnik and Yoshie, 2000, 2012). Four chemokine subfamilies, CXC, CC, XC, and CX3C, were categorized according to the arrangement of first two conserved cysteine residues in the sequence of amino acid. The two largest subfamilies which include at least 50 chemokines consist of the CXC and CC chemokines. In the CXC chemokines, there is an amino acid residue between two cysteine residues, and in the CC chemokines, there are two cysteine adjacent residues (Zlotnik and Yoshie, 2000; Bacon *et al*., 2002; Alejo and Tafalla, 2011). Moreover, CC chemokines predominantly induce monocytes, but some also have potent chemotactic activity against lymphocytes and eosinophils (Kuroda *et al*., 2003). Few chemokines have been found in the other two subfamilies. Recently, all chemokines have been categorized into four other types based on their function, these include I type (inflammatory chemokines), H type (homeostatic chemokines), D type (dual chemokines), and P type (plasma or platelet chemokines activated

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by cleavage). However, some chemokines remain uncategorized because their function remains unknown. Chemokines are secreted by neutrophils, monocytes, and other cells following the pathogen recognition receptors to stimulate leukocyte migration by interacting with seven trans-membrane G protein-coupled receptors to the site of injury or infection (Zlotnik and Yoshie, 2000; Yoshie et a1., 2001). Evolutionarily, fish are an intermediate between species which rely only upon an innate immune system, and those which are mainly dependant upon an aquired immune system as mechanisms of pathogen defence (He *et al*., 2004). Thus, the study of fish chemokines can be used to both improve the use of chemokines to enhance the congenital immune response and disease resistance, as well as study the mechanisms of innate immune activation in this species.

To further study the expression profiles of CC family members and the immune-regulatory mechanisms of large yellow croaker, this study reports the molecular characteristics and expression profile of three genes (dual chemokine ligand CCL17 and homeostatic chemokines ligands CCL21 and CCL24) following bacterial challenge.

MATERIAL AND METHODS

Fish rearing, bacterial challenge and tissue sampling

Healthy large yellow croaker were collected from the Shacheng Harbor Cultivation Base (Fujian Province, China) and immediately transferred to the laboratory. A total of 90 *L. crocea* individuals (average body weight: 180 g) were obtained to study the effect of bacterial challenge on CCL expression. All individuals were rested in 20°C circulating seawater. The fish were fed for two weeks to adapt to the indoor cultural environment. *Vibrio anguillarus* was obtained from the Institute of Marine Fisheries Microbiology Laboratory (Zhejiang, China). The cryopreserved strain was resurrected by drawing a "Z" line and cultured in a 28°C incubator. Then, a single bacteria colony was selected. The *V. anguillica* challenge was performed as follows: the bacteria were mixed in sterile PBS (pH 7.4); after acclimatization, 30 fish were randomly sampled as a control group, with an intraperitoneal injection of PBS (pH 7.4; 300 µL), a second group of 30 individuals were intraperitoneally injected with poly I:C (300 µL), and the remaining 30 were intraperitoneally injected with *V. anguillarus* (1×10^8 CFU/mL, resuspended in PBS, pH 7.4) for the bacterial challenge experiment; after injection, the fish were released back into the tank. After 24 h, the liver, kidney, spleen, muscle, heart, brain, intestine, and gill of each fish from five randomly chosen individuals in each group were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction

The stored tissues in the control group were used for RNA extraction first. The tissues in the poly I:C and bacterial groups were selected to perform further processes after the analysis of tissue expression in control group. Then, the liver, kidney, and spleen tissues were further subjected to RNA extraction that three target genes express well in the three tissues. TRIzol Reagent (Invitrogen, USA) was used to extract the total RNA from all of the selected tissues. RNA was treated with RNase-free DNase I (TaKaRa, Japan) and then synthesized into cDNA using a first strand cDNA synthesis kit (Toyobo, Japan) according to the manufacturer's protocol. The cDNA samples were stored at -20°C for further expression analysis (Cui *et al.,* 2017).

Identification of complete ORF, molecular characteristics, and phylogenetic analysis of CCLs

The complete ORF sequence of CCL14, CCL21, and CCL24 was obtained from the *L. crocea* whole-genome data (Wu *et al*., 2014) and submitted to the Genbank database (MG253868, MG253869, and MG253870). The amino acid sequence of the CCLs genes were translated by DNAMAN V6.0 and aligned with other species by GeneDoc. A nucleotide homology search was conducted using the BLASTn program (http://www.ncbi.nlm.gov/BLAST/). A phylogenetic tree was reconstructed by Molecular Evolution Genetics Analysis (MEGA) software version 5.0, using the Maximum Parsimony method.

qRT-PCR and expression analysis

Three primer pairs for each of the three genes were designed based on the complete ORF sequence of CCL14, CCL21, and CCL24 for quantitative real-time PCR (qRT-PCR) (Table I) and β-actin was selected as the internal control for the CCL mRNA expression analysis (Dong *et al*., 2016). The qRT-PCR process and expression analysis have been reported in our previous work (Dong *et al*., 2016).

Table I.- qRT-PCR primers for CCLs and β-actin.

Primers	Sequences (5'-3')	Product size
CCL17-F	CGCTCTCCTGTGCATCCT	229bp
CCL17-R	CTTCACCCACTTGTCGTCC	
CCL21-F	TTCGTTCTCCTGTTCCTCAC	242bp
CCL21-R	TCCTGGACCCAAAGTTCATT	
CCL24-F	ATCCAACCAGGACACCCACC	188bp
CCL24-R	CTCCGACTCGACACCGCC	
β-actin-F	TTACTCCTTCACCACCACAG	232 bp
β-actin-R	ATTCCGCAAGATTCCATAC	

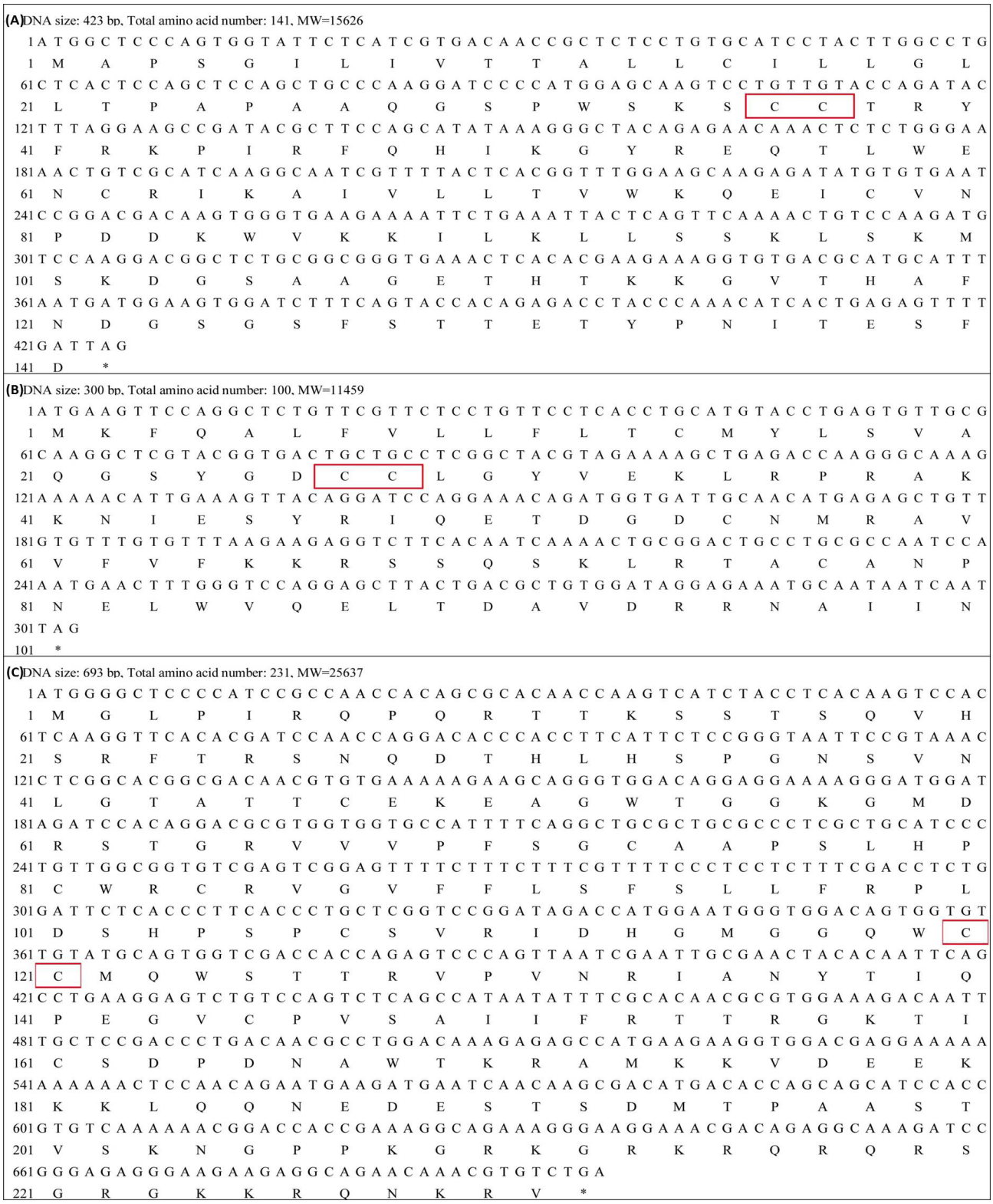

Fig. 1. The complete cDNA ORF of CCL17 (A), CCL21 (B) and CCL24 (C). Red, the first two conserved cysteine residues C-C position for each gene.

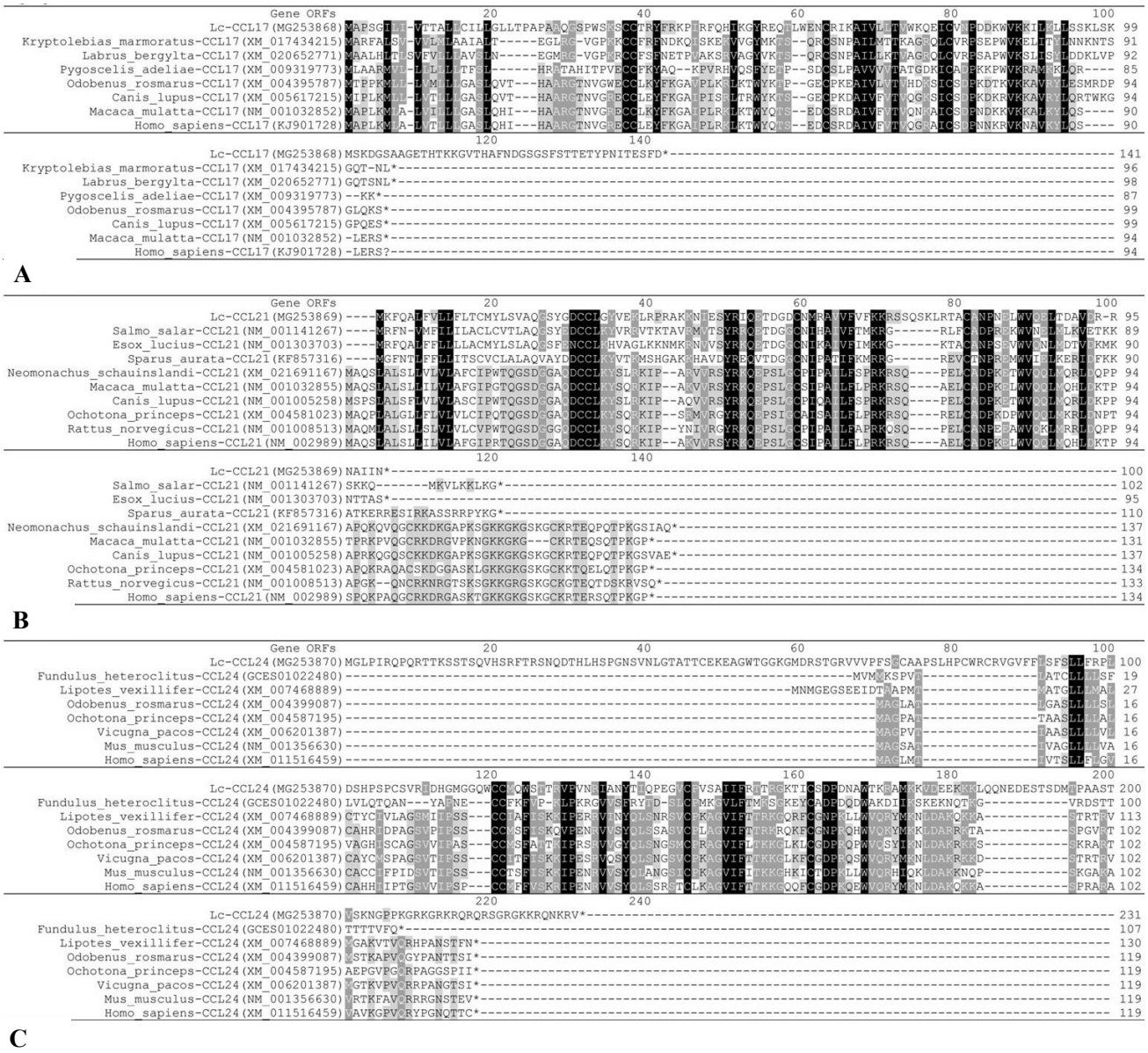

Fig. 2. Multiple alignment of the deduced amino acid sequences of CCL17 (A), CCL21 (B) and CCL24 (C) from *L. crocea*. Note: A question mark at the end of human CCL17 AA sequence means missing of a terminator on the DNA level from the annotation of Genbank Database.

RESULTS

CCL cDNA sequence analysis and characterization of the complete cDNA ORFs

The 423 bp ORF of CCL17 (Fig. 1A) encodes 141 amino acid (AA) residues. In the AA sequence, the first two conserved cysteine residues (C-C) are present at the 36th-37th position. CCL21 (Fig. 1B) encodes a relatively smaller ligand than CCL17 and CCL24, which encodes 231 AA residues. The first C-C region is present at the 27th-28th position for CCL21 and 120th-121st position for CCL24 (Fig. 1B, C).

The AA sequence of CCL17 from the croaker was aligned with that of humans (*Homo sapiens*), walrus (*Odobenus rosmarus divergens*), dogs (*Canis lupus familiaris*), monkeys (*Macaca mulatta*), penguins (*Pygoscelis adeliae*), and other fish (*Kryptolebias marmoratus,* and *Labrus bergylta*) (Fig. 2A). Croaker CCL17 codes 141 AAs, which is the longest AA chain among the seven chosen species; the other CCL17s

encode an 87-99 AA chain. This indicates that the Lc-CCL17 gene codes a larger ligand than other species. Two conserved leucine residues upstream of the CC region and eight additional downstream AA residues were found in the CCL17 AA sequences (Fig. 2A). Compared with the other fish species, there are more conserved AA residues compared to the mammals, as represented in the evolution analysis below. The AA sequence of croaker CCL21 was aligned with that of humans (*Homo sapiens*), dogs (*Canis lupus familiaris*), monkeys (*Macaca mulatta*), seals (Neomonachus schauinslandi), rats (*Rattus norvegicus*), pika (*Ochotona princeps*), and other fish species (*Salmo salar, Esox lucius,* and *Sparus aurata*) (Fig. 2B). Croaker CCL21 codes 100 AAs, which is highly similar to the three other fish species (102 AA for *S. salar*, 95 AA for *E. Lucius* and 110 AA for *S. aurata*), and differs compared to the mammals (131-137 AA). A conserved Asp-Cys-Cys-Leu fragment was found upstream of the AA chain and followed by a conserved Tyr-Arg-X-Gln fragment. For the "X" of the Tyr-Arg-X-Gln fragment, Lys is the common counterpart for all mammals and four AA differ between the four fish species, indicating a higher rate of variability in CCL21 in fish compared to that of mammals. The AA sequence of CCL24 was aligned with that of humans (*Homo sapiens*), mice (*Mus musculus*), walrus (*Odobenus rosmarus divergens*), pika (*Ochotona princeps*), alpaca (*Vicugna pacos*), dolphins (*Lipotes vexillifer*), and other species of fish (*Fundulus heteroclitus*) (Fig. 2C). The length of the Lc-CCL24 AA chain (231 AA) is double that of the other compared animals (107-130 AA). A 60-70 AA fragment of Lc-CCL24 was isolated in the alignment of eight animals. A Leu-Leu conserved AA residue was found upstream of Cys-Cys conserved fragment, and compared with CCL17 and 21, there were fewer conserved AA fragments in the CCL24 AA chain.

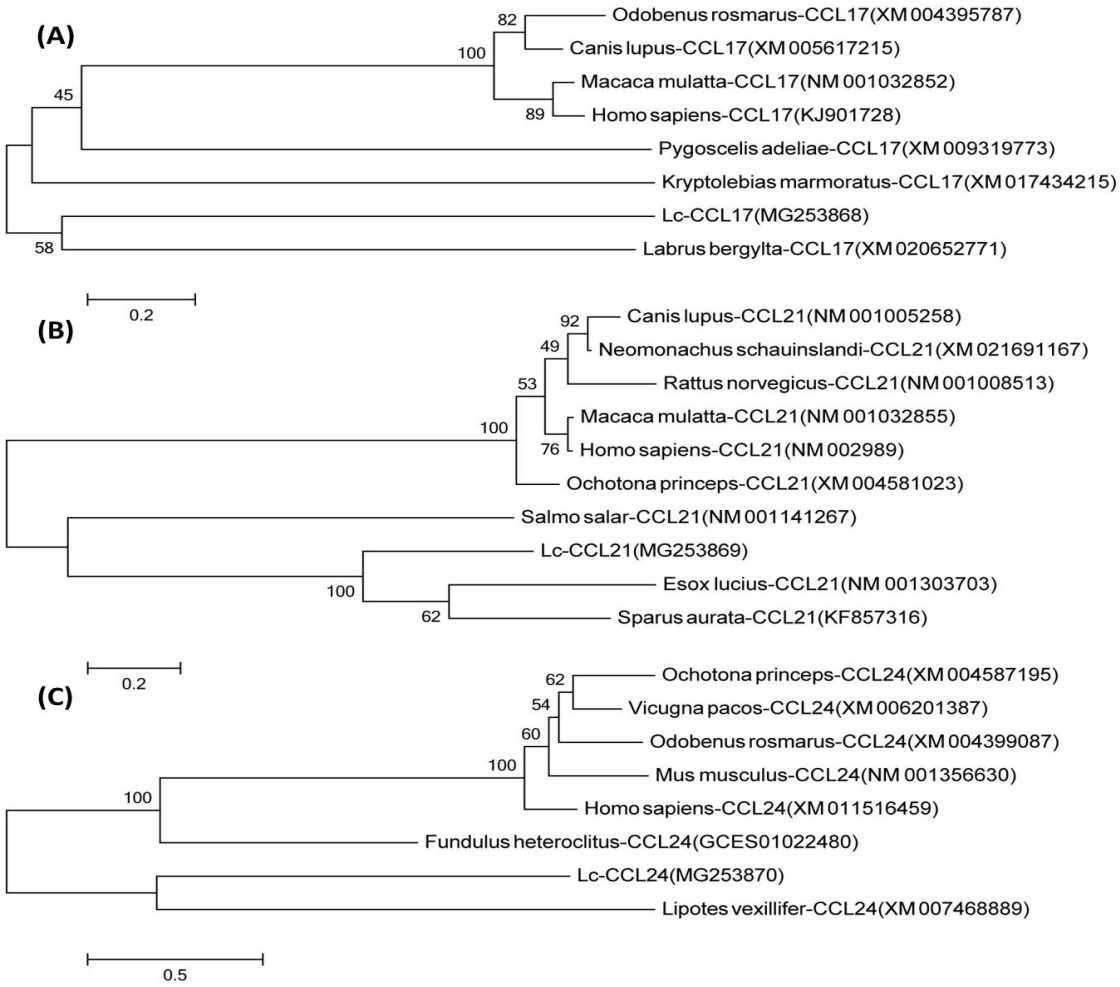

Fig. 3. Phylogenetic tree of the amino acid sequences of CCL17 (A), CCL21 (B) and CCL24 (C) from *L. crocea*.

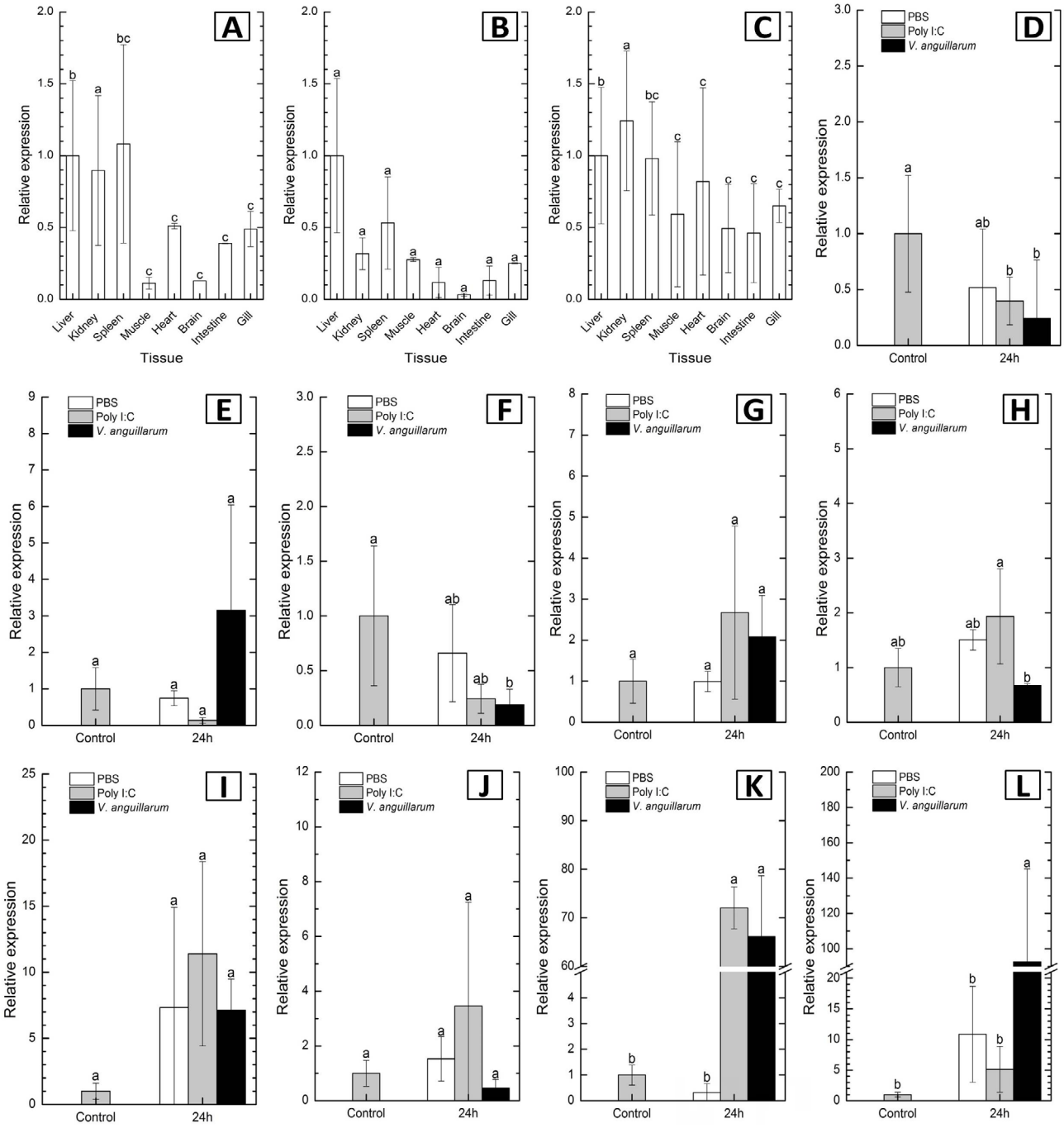

Fig. 4. Expression analysis of the CCLs, (A) CCL17, (B) CCL21 and (C) CCL24, in various tissues in uninfected fish and challenged liver (D, G and J for CCL17), kidney (E, H and K for CCL21) and spleen (F, I and L for CCL24) by Poly I:C and *V. anguillica*.

Phylogenetic analysis

To reveal the molecular phylogenetic position of the CCLs, an unrooted phylogenetic tree based on chemokines was constructed using MEGA v5.05 via the neighbor-joining method (Fig. 3). The results revealed that different classes of chemokines formed their own separate branches. The bootstrap value at the nodes of each class was above 70%, demonstrating that those nodes provided no

significant differentiation. The phylogenetic relationship of the CCLs was based on the multiple alignments of the CCLs from mammals, birds, and fish. The phylogenetic tree showed that the CCL17 gene formed the teleost clade with *Kryptolebias marmoratus* and *Labrus bergylta* (Fig. 3A). Moreover, the fish clade was clustered together with the mammalian clades (Fig. 3A). For CCL21, the tree exhibited a similar clade model with that of CCL17 (Fig. 3B). It is interesting to note that the clade for croaker CCL24 was grouped together with dolphin first, followed by a fish clade (Fig. 3C).

The temporal expression profile of CCL mRNA after V. anguillarum *challenge*

To detect the potential function of the three CCLs in *L. crocea*, the tissue distribution of the mRNA expression pattern was analyzed for each CCL. The three molecules exhibited broad tissue distribution, with differential levels of expression in the liver, spleen, kidney, muscle, heart, brain, intestine, and gills (Fig. 4). Relatively higher levels of expression for the three genes were observed in the liver, spleen, and kidney of healthy fish compared to the other tissues (Fig. 4). The relative expression levels of CCL17 in the liver and kidney were significant different compared to that of the other five tissues ($p < 0.05$; Fig. 4A), and similar observations were found for CCL24 ($p < 0.05$; Fig. 4C). However, no significant differences were observed between the three immune organs (liver, spleen, and kidney) and the other five tissues (muscle, heart, brain, intestine, and gills) for the relative level of CCL21 expression ($p > 0.05$; Fig. 4B). Following pathogenic bacteria (*V. anguillarum*) challenge, the temporal expression profile exhibited a clear and explicit expression level of CCLs mRNA in the liver, spleen, and kidney at 24 h (Fig. 4D-L). Figure 4D-F presents the CCL17 gene expression profile in the liver (Fig. 4D), kidney (Fig. 4E), and spleen (Fig. 4F) following bacteria and poly I:C challenge, compared to the PBS control. Compared with the control, the expression of CCL17 were significantly downregulated in the liver and spleen ($p < 0.05$) and was upregulated in the kidney ($p > 0.05$) after challenge (Fig. 4D-F). No significant difference between the control and bacteria-challenged groups was found for the CCL21 gene in all three tissues (Fig. 4 G-I). Compared to CCL17 and 21, a different expression profile was found for CCL24. Figure 4K and L shows the significant upregulation ($p < 0.05$) in the kidney and spleen after challenge. Three genes and three tissues presented different CCL expression profiles.

DISCUSSION

The CC-type ligands CCL17, CCL21, and CCL24 are considered dual chemokine and homeostatic chemokines, respectively. These two functional definitions are derived from the four functional distributions of chemokine ligands described in previous studies (Zlotnik and Yoshie, 2012). Seven ligands, CCL11, CCL17, CCL20, CCL22, XCL1, XCL2, and CX3CL1, were categorized as dual-function chemokines, which have both inflammatory and homeostatic functions. CCL22 and CCL17 share the same receptor, CCR4, which is expressed by T cells in inflamed tissues. CCL17 is expressed by microvascular endothelial cells in the skin, different with CCL22 by dermal dendritic cells. However, the CCL17 ligand interacts with CCR4 on T cells before CCL22 (Mariani *et al*., 2004). In contrast, the two other ligands, CCL21 and 24, have the sole function of homeostasis, a role which includes regulating the movement and localization of lymphocyte and dendritic cell subsets in the immune system (Moser *et al*., 2004). The receptor for CCL21 is CCR7, which functions in the homing of lymphocytes and dendritic cells to the secondary lymphoid tissues. CCR7 is expressed in the T cell zone of secondary lymphoid tissues and high endothelial venules (HEV). Thus, CCL21 guides lymphocytes and dendritic cells expressing CCR7 into the lymph nodes and T cell zones via HEVs and afferent lymphatics. A similar function of CCL24 was reported in previous studies, in response to CCR3 (Zlotnik and Yoshie, 2012).

The three phylogenetic trees revealed the molecular phylogenetic position of each CCL in the current study. CCL17 and CCL21 formed in the teleost clade first and then clustered within the mammal clades, which aligns with the findings of Peatman and Liu (2007). In contrast, CCL24 differed in that the dolphin clade was associated with the teleost clade. There is insufficient evidence to support this unusual result, and it should be further studied.

The results of the three CCL mRNA expression patterns revealed higher levels of expression in the immune tissues of the spleen, kidney and liver. This indicates that all three genes are related to the immune response of fish. However, skin mRNA could not be analyzed in the current study, which is considered a limitation since the CCL17 is expressed by microvascular endothelial cells in the skin tissue. Following the challenge with pathogenic bacteria (*V. anguillarum*), which induced an inflammatory response in the immune tissues, the level of CCL17 mRNA expression in the liver and spleen was downregulated significantly ($p < 0.05$), and upregulated in the kidney ($p > 0.05$). This observation could be explained by the dual function of CCL17. Similarly, the lack of a significant change in the expression of CCL21 between the control and bacterial-challenged fish, and the significant upregulation of CCL24 ($p < 0.05$) in the kidney and spleen could be evidence of the homeostatic function of these CCLs. Taken together,

our findings indicate that the immunological effect of CCLs in large yellow croaker is weaker than that of other fish species (Alejo and Tafalla, 2011), which may be a mechanism of increased infectability of the large yellow croaker (Wu *et al*., 2014).

CONCLUSION

This study briefly reports the immune expression profiles of the dual chemokine, CCL17, and homeostatic chemokines CCL21, and CCL24 in the large yellow croaker. The relationship between the expression of additional chemokines and the involvement of the *L. crocea* immune response requires further study. Such findings may be valuable for the development of methods of disease resistance in the aquaculture industry.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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